ANALYSIS OF NUCLEOTIDE VARIANTS IN THE PROMOTER REGION OF THE URIDINE DIPHOSPHATE GLUCURONOSYLTRANSFERASE 1A1 (*UGT1A1*) GENE AMONG NEONATAL JAUNDICE MALAY INFANTS WITHOUT VARIATIONS IN THE EXONIC REGIONS

By

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TABLE OF CONTENTS

Acknowledgement ii

Table of contents iii

List of tables viii

List of figures x

List of appendices xiii

List of abbreviations xiv

List of symbols xvii

Abstrak xviii

Abstract xx

CHAPTER 1 INTRODUCTION

1.1 Neonatal hyperbilirubinemia 1

1.2 Uridine Diphosphoglucuronate Glucuronosyl Transferase (UDPGT) 1A1 Gene 2

1.3 Gilbert syndrome 8

1.4 CriglerNajjar syndrome 9

1.5 Bilirubin Uridine Diphosphate Glucuronosyltransferase (B-UDPGT) 11

1.51 Indirect Hyperbilirubinemia (Unconjugated Bilirubin) 12

1.52 Direct Hyperbilirubinemia (Conjugated Bilirubin) 12

1.53 Redox cycle 13

1.6 Mechanism of Phototherapy 16

1.7 Bilirubin metabolism 23

1.8 Single Nucleotide Polymorphism 29

1.10 Principle of High Resolution Melting Analysis 30

1.11 Objective(s) 33

1.11.1 Aim of study 33

1.11.2 Specific objectives of the study 33

CHAPTER 2 MATERIAL & METHODS

2.1 Materials 34

2.1.1 Subject 34

2.1.2 Sample size (obtained from previous studies) 37

2.1.3 High Resolution Melting Analysis reagent 38

2.1.3.1 DNA sample 38

2.1.3.2 Precision melt supermix 38

2.1.4 Polymerase Chain Reaction (PCR) 39

a. Gene Amp 10X PCR buffer II (100mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCI_2$ and 0.0.1% (w/v))(Applied Biosystems, California, USA) 39
b. 10mM dNTP blend 39
c. Gene Amp 25mM Magnesium dichloride ($MgCI_2$)(Applied Biosystem, California, USA) 39
d. 5U/µl of amplitaq gold DNA polymerase (250 units)(Applied Biosystem, California, USA) 39
e. Oligonucleotide primers (Helix Biotech (M) SdnBhd) 39

2.1.5 Gel electrophoresis 40

a. Preparation of 10X Tris Base EDTA (TBE) buffer 40
b. Agarose powder 40
c. 6X Blue loading dye 40

d. SYBR stain (SYBR® Green I) 41
e. DNA ladder (100bp) 41
2.2 Methods 41
2.2.1 DNA quantification 41
a) NanoQuant 41
2.2.2 High Resolution Melting Analysis (HRMA) 42
2.2.3 Polymerase Chain Reaction (PCR) analysis 53
2.2.4 Agarose gel electrophoresis 56
2.2.5 PCR purification 56
2.2.6 DNA sequencing 57
2.2.7 Statistical analysis 57

CHAPTER 3 RESULTS

3.1 Baseline data 58
3.2 Genomic DNA selection 60
3.3 DNA quantification 61
3.4 Amplification of *UGT1A1* gene 63
3.5 High Resolution Melting (HRM) Analysis 65
3.5.1 The distribution of c.-3279T>G variant 66
3.5.1.1 The distribution of c.-3279T>G variant jaundiced and non-jaundice group 66
3.5.1.2 High Resolution Melting result for c.-3279T>G variant 67
3.5.1.3 Sequencing result for c.-3279T>G 68
3.5.2 The distributionof TATA BOX variant 69
3.5.2.1 The distribution of TATA BOX variant in jaundiced and non-jaundice group 69

3.5.2.2 HRM result for variant TATA BOX 70

3.5.2.3 Sequencing result for TATA box 71

3.5.3 The distribution of c.-3156G>A variant 72

3.5.3.1 The distribution of c.-3156G>A variant in jaundiced and non-jaundice group 72

3.5.3.2 HRM results for variant C.-3156G>A 73

3.5.3.3 Sequencing result for c.-3156G>A 74

3.5.4 The distribution of c.-64G>C and CAAT box variant 75

3.5.4.1 Bar chart data for c.-64G>C 75

3.5.4.2 HRM results for variant c.-64G>C 76

3.5.4.3 Sequencing result for c.-64G>C 77

3.7 Sequencing results 82

3.8 Summary of the promoter region *UGT1A1* gene analysis 82

CHAPTER 4 DISCUSSION

4.1 Clinical Data 83

4.2 Primer design in promoter region of the *UGT1A1* gene 83

4.3 The c.-3279T>G variant 84

4.3.1 c.-3279T>G variant: Frequency 84

4.3.2 c.-3279T>G variant: Association with jaundice 85

4.4 Other variant in the promoter region of the *UGT1A1* gene 86

a) A(TA)NTAA in the TATA box variant 86

b) c.-3156G>A variant 89

c) CAAT box variant 90

d) c.-1126 C>T variant 91

e) c.-64G>C variant 92

4.5 Linkage Disequilibrium 93

4.6 Strength of the study 96

4.7 Benefit of this study 96

4.8 Recommendations for future research 96

CHAPTER 5 CONCLUSION 98

REFERENCES 99

APPENDICES 108

LIST OF TABLES

		Page
Table 1.1	Comparison disorder of unconjugated hyperbilirubinemia Rewrite from:GourleyGR. Neonatal jaundice and disorder of bilirubin metabolism. Suchy FJ, Sokol RJ, BalisteriWF [Eds]. Liver disease in children, 3rd ed. New York, Cambridge University Press, 2007	10
Table 2.1	Sequence of primers for HRM analysis	44
Table 2.2	HRM mastermix composition for c.-3279T>G analysis	45
Table 2.3	HRM protocol for precision melt supermix of the c.-3279T>G analysis	46
Table 2.4	HRM mastermix composition for c.-3156G>A analysis	47
Table 2.5	HRM protocol for precision melt supermix of the c.-3156G>A analysis	48
Table 2.6	HRM mastermix composition for TATA BOX analysis	49
Table 2.7	HRM protocol for precision melt supermix of the TATA BOX analysis	50
Table 2.8	HRM mastermix composition for c.-1126 C>T	51
Table 2.9	HRM protocol for precision melt supermix of the c.-1126 C>T analysis	52
Table 2.10	Total volumes of reagents used in PCR amplification of promoter*UGT1A1* gene	54
Table 2.11	Melting temperature and size of PCR product for each sequencing primer	55
Table 3.1	Mean±SDs values for three type of variant in the TATA box of *UGT1A1* gene	78
Table 3.2	Distribution of genotypes and allele frequencies of the promoter region *UGT1A1*gene among the jaundice andnon-jaundice sample group among Malaysian neonates. NA: not applicable, a: chi square test applied, b:Fisher exact test applied	79

Table 3.3 Association status of common haplotype of promoter region of *UGT1A1* gene. The *p*-value was set up at <0.05. For the first arrangement of nucleotide indicate variant of c.-64G>C, followed by c.-3279 T>G, c.-3156 G>A dan TATA BOX 81

LIST OF FIGURE

		Page
Figure 1.1	Location of *UGT1A1* gene in chromosome 2 (2q37.1)	4
Figure 1.2	Sites for Human uridinediphosphate glucuronosyltransferase-1 gene (UGT1). UDPGA, uridine diphophateglucuronic acid binding site and membrane spaning region.	5
Figure 1.3	The complete scheme of the stereochemistry biosynthesis of bilirubin metabolism. The production of bilirubin from hemecatalyse by heme-oxygenase and subsequently biliverdin reductase. Glucuronidation of bilirubin by UDP glucuronosyltransferase *UGT1A1* results in the formation of bilirubin mono- and di-glucuronide which are excreted into bile.The figure was adapted from (Bosma, 2003)	15
Figure 1.4	Biological pathway of normal bilirubin metabolism with and without phototherapy (Maisels and McDonagh, 2008)	17
Figure 1.5	Mechanism absorption of light by normal form bilirubin (McDonagh,1986)	19
Figure 1.6	The stereochemistry of 2 isomeric form; structural and configurational isomer structure when absorbtion of light by dermal surface. Taken from: Gourley GR. Neonatal jaundice and disorder of bilirubin metabolism. Suchy FJ, Sokol RJ, Balisteri WF [Eds]. Liver disease in children, 3rd ed. New York, Cambridge University Press, 2007	20
Figure 1.7	Bilirubin diglucuronide. In bilirubin monoglucuronide, only one propionic acid side chain (C-8 or c-12) is glucuronidated. Taken from: Gourley GR. Neonatal jaundice and disorder of bilirubin metabolism. Suchy FJ, Sokol RJ, Balisteri WF [Eds]. Liver disease in children, 3rd ed. New York, Cambridge University Press, 2007	22
Figure 1.8	Three possible chemical structures-the conversion of heme to bilirubin.	26
Figure 1.9	High Resolution Melting Analysis Machine	32
Figure 2.1	Flowchart of the study design	36
Figure 2.2	Sequence of c.-3279T>G variant	43
Figure 2.3	Sequence of c.-3156G>A variant	43

Figure 2.4	Sequence of c.-1126 C>T variant	43
Figure 2.5	Sequence of TATA BOX variant	44
Figure 3.1	Sex distribution between jaundiced and non-jaundice groups	59
Figure 3.2	The electrophoresis of DNA genomic for jaundiced and non-jaundice sample. The thickness of the bands indicates high amount of DNA yield in the sample. DNA samples were obtained from the whole blood and extracted using QIAGEN blood mini kit.	62
Figure 3.3	Gel electrophoresis of PCR product for each fragment of interest or primer in the promoter region of the *UGT1A1* gene	64
Figure 3.4	Results for c.-3279 T>G. Homozygous wild type (T/T), Heterozygous (T/G) and Homozygous variant(G/G)	66
Figure 3.5	High resolution melting analysis of c.-3279 T>G variant in the *UGT1A1* gene. HRM graft and difference graph are shownpattern above. Homozygous wild type (T/T), Homozygous (G/G) and Heterozygous (T/G).	67
Figure 3.6	Sequencing result for Homozygous wild type (T/T), Homozygous variant (G/G) andHeterozygous (T/G)	68
Figure 3.7	Results for TATA box. Homozygous wild type (A(TA)6TAA/A(TA)6TAA), Homozygous variant (A(TA)7TAA/A(TA)7TAA) and Heterozygous (A(TA)6TAA/A(TA)7TAA).	69
Figure 3.8	High resolution melting analysis for TATA box variant in the *UGT1A1* gene. HRM graft and difference graph shown different pattern above. Homozygous wild type (A(TA)6TAA/A(TA)6TAA), Homozygous variant (A(TA)7TAA/A(TA)7TAA) and Heterozygous (A(TA)6TAA/A(TA)7TAA).	70
Figure 3.9	Sequencing results for Homozygous wild type (A(TA)6TAA/A(TA)6TAA), Homozygous variant (A(TA)7TAA/A(TA)7TAA) and Heterozygous (A(TA)6TAA/ A(TA)7TAA)	71
Figure 3.10	Results for c.-3156 G>A. Homozygous wild type (G/G), Heterozygous (G/A) and Homozygous variant (A/A)	72

Figure 3.11	High resolution melting analysis of c.-3156 G>A variant in the *UGT1A1* gene. HRM graft and difference graph are shown different pattern above. Wild type (G/G), Homozygous (A/A) and Heterozygous (G/A).	73
Figure 3.12	Sequencing result for Homozygous wild type c.-3156 (G/G), Heterozygous (G/A) and Homozygous variant (A/A)	74
Figure 3.13	Results for c.-64 G>C. Homozygous wild type (G/G) and Heterozygous (G/C)	75
Figure 3.14	High resolution melting analysis of c.-64 G>Cin the *UGT1A1* gene. HRM graft and difference graph are shown different pattern above. Wild type (G/G), Homozygous (G/C)	76
Figure 3.15	Sequencing result for Homozygous wild type c.-64 (G/G) and Heterozygous (G/C)	77
Figure 3.16	Linkage disequilibrium plot. Haplotype frequencies and LD were calculated using Haploview software (version 4.2). The color of cell represented the strength of LD between two markers and the rs numbers are extracted from the Ensembl database. The loci rs4124874 and rs10929302 are in intermediate LD meanwhile no significant LD was found in rs4124874 and rs8873478 and also loci rs10929302 and rs8873478	82

LIST OF APPENDIX

Appendix		Page
Appendix A	Publications and presentations	108

LIST OF ABBREVIATIONS

µg/µl	: Microgram per microlitre
µl	: Microlitre
µM	: Micro Mol
µmol/L	: Micromol per litre
A	: Adenine
A260/A280	: Ratio of 260 absorbance over 280 absorbance
ABO	: Types of blood group: A, B, O and AB
BBB	: Blood brain barrier
Bp	: Base pair
Buffer AE	: Elusion buffer
Buffer AW	: Wash buffer
Buffer BL	: Lyses buffer
Buffer EB	: Elusion buffer
Buffer PB	: Purification buffer
B-UGT	: Bilirubin uridine diphosphate-glucuronosyltransferase
C	: Cytosine
c.	: Coding number
cDNA	: Complementary DNA
CN	: CriglerNajjar syndrome
CNI	: CriglerNajjar syndrome type I
CNII	: CriglerNajjar syndrome type II
CNS	: Central nervous system
ddH_2O	: Deionized distilled water
dNTP	: Modified nucleotides
DMSO	: Dimethylsulfoxide

DNA	: Deoxyribonucleic acid
DNTP	: Dinucleotide triphosphate
dsDNA	: Double strand DNA
DTTP	: Deoxythymine triphosphate
EDTA	: Ethylenediaminetetraacetic acid
ER	: Endoplasmic reticulum
G	: Guanine
G6PD	: Glucose-6-phosphate dehydrogenase
G71R	: Glycine to argine at codon 71
GS	: Gilbert syndrome
Hb	: Hemoglobin
HO	: Heme oxygenase
Kb	: Kilo base pair
Kg	: Kilogram
M_1V_1	: Mol volume
$MgCl_2$	: Magnesium Chloride
ml	: Millilitre
ml/min	: Milliliter per minute
µM	: Micro mol
mm	: Millimeter
mRNA	: Messenger RNA
N	: Sample size
NADPH	: Nicotinamide adenine dinucleotide phosphate
NCBI	: National Centre of Biotechnology Informatics
ng/µl	: Nanogram/microlitre
NNJ	: Neonatal jaundice
NW	:Non-wash

O_2	: Oxygen
PCR	: Polymerase chain reaction
RBC	: Red blood cell
RFLP	: Restriction Fragment Length Polymorphism
RNA	: Ribonucleic acid
Rpm	: Round per minute
SB	: Serum bilirubin
SD	: Standard deviation
SNP	: Single nucleotide polymorphism
SPSS	: Science package social software
SSCP	: Single strand conformation polymorphism
T	: Thymine
TBE	: Tris base EDTA
TEAA	: Triethylammonium acetate
TSB	: Total Serum Bilirubin
U	: Urasil
U/µl	: Unit per microliter
UDPGT	: Uridine diphosphoglucuronosyltransferase
UGT	: Uridine glucuronosyltransferase
UGT1A1	: Uridine glucuronosyltransferase 1A1 isoform
USM	: Universiti Sains Malaysia
UV	: Ultra violet
ρ	: Piko

LIST OF SYMBOLS

∞	: Infiniti
<	: Less than
>	: More than
ºC	: Degree celcius
~	: Approximately
G	: Gram
A	: Alpha
B	: Beta
%	: Percentage
/	: or
±	: Plus minus
P1	: Proportion of the G71R variant in non jaundiced babies
P2	: Proportion of the G71R variant in jaundiced babies
Zα	: Value of the standard normal distribution cutting of probability α
Zβ	: Value of the standard normal distribution cutting of probability β

ANALISA VARIASI JUJUKAN DNA DI DALAM BAHAGIAN PERMULAAN GEN URIDINE DIPHOSPHATE GLUCURONOSYLTRANSFERASE 1A1 (*UGT1A1*) DI KALANGAN BAYI KUNING TANPA VARIASI DI BAHAGIAN EXONIC

ABSTRAK

Pengeluaran bilirubin yang berlebihan merupakan salah satu faktor yang menyebabkan demam kuning dalam kalangan bayi baru lahir dan proses ini dimangkinkan oleh sejenis enzim iaitu Uridine glucuronosyltransferase yang dikodkan oleh gen *UGT1A1* dan ini adalah suatu keadaan yang lazim berlaku selepas lahir. Objektif kajian ini adalah untuk mengesan kehadiran mutasi pada bahagian permulaan gen *UGT1A1* dalam kalangan bayi Melayu yang menghidap demam kuning dan tanpa demam kuning dengan menggunakan kaedah analisis resolusi tinggi lebur (HRM) dan untuk mencari hubungkait antara mutasi-mutasi yang dikenal pasti dengan menggunakan analisis ketidakseimbangan. Semua sampel yang bebas dari mutasi pada bahagian ekson telah diambil daripada sambungan kajian Nur Hasnah Ma'amor. Analisis HRM dilakukan bagi menyaring bahagian permulaan gen *UGT1A1* gene dan subjek yang dikenal pasti mengandungi bentuk yang berbeza semasa analisis HRM akan menjalani proses penjujukan DNA bagi mengesahkan kehadiran mutasi. Data dianalisa dengan menggunakan SPSS program. Seramai 250 bayi yang menjalani fototerapi dikategorikan sebagai kumpulan bayi kuning dan seramai 260 bayi yang tidak melalui fototerapi dikategorikan sebagai bayi normal. Enam mutasi telah dianalisis dalam kajian ini dan dua mutasi tidak berjaya ditemui

dalam populasi Melayu. Mutasi yang paling kerap adalah c.-3279T>G yang terletak pada bahagian permulaan gen *UGT1A1*. Semua mutasi ini pernah dilaporkan dalam populasi India, Caucasian dan Jepun. Seramai 163 pesakit mempunyai variasi c.-3279T>G telah dikenal pasti dengan nilai p=0.001 dan ianya merupakan faktor berlakunya demam kuning di kalangan bayi Melayu.

ANALYSIS OF NUCLEOTIDE VARIANTS IN THE PROMOTER REGION OF THE URIDINE DIPHOSPHATE GLUCURONOSYLTRANSFERASE 1A1 (*UGT1A1*) GENE AMONG NEONATAL JAUNDICE MALAY INFANTS WITHOUT VARIANTS IN THE EXONIC REGIONS

ABSTRACT

Overproduction of bilirubin is one of the major factor which contributes to neonatal hyperbilirubinemia that is catalyzed by an enzyme Uridine glucuronyl transferase encoded by the *UGT1A1* gene. This is a normal physiological occurance after birth. The objectives of this study were to i) determine the presence of variants or polymorphisms in the promoter region of the *UGT1A1* gene amongst Malay infants with and without jaundice using high resolution melting (HRM) analysis and to assess the correlation between identified variants or polymorphisms using linkage disequilibrium analysis. All samples that were free from variant in the exonic regions were recruited from another study by Hasnah Ma'amor in year 2011. High resolution melting analysis was performed to screen for the promoter region in the *UGT1A1* gene and for subjects who were identified to have different pattern of HRM analysis, sequencing was performed to confirm the presence of variant. SPSS was used for data analysis. Two hundred and fifty (250) infants who were undergone phototherapy and 260 infants who were not undergoing phototherapy were included in the jaundiced and non-jaundice groups. Six variants were investigated but two were not detected in this study. The most common variant was c.-3279T>G which is common in other Asian populations as well. All the variants detected in this study have been

reported as SNPs in Indian, Caucasion and Japanese populations. The c.-3279T>G variant had been identified as the most common variant in 163 jaundice Malay patientsin the promoter region of the *UGT1A1* gene with the *p*-value 0.001 and is a risk factor for Malay neonatal hyperbilirubinemia.

CHAPTER 1

INTRODUCTION

1.1 Neonatal hyperbilirubinemia

Neonatal hyperbilirubinemia is a normal physiological condition after birth and is a major concern for paediatricians and parents. This phenomenon is related to the deposition of serum bilirubin in the tissue leading to yellow discoloration of skin, mucosal membranes and sclera in newborn neonates.

According to Maisels 1988, this phenomenon occurs in 60% of full term infants and 80% of premature infants (Maisels *et al.*, 1988). Based on a study in 2002, the peak serum bilirubin level among full term Asian (Japanese, Korean and Chinese) population are double compared to the Caucasian and Black population (Avery, 1981; Avery *et al.*, 2005). In addition to this, the frequency and severity of neonatal jaundice in Asians are higher compared to Caucasians, including the incidence of kernicterus (Avery, 1981; Fischer *et al.*, 1988; Setia *et al.*, 2002).

The incidence of neonatal hyperbilirubinemia is also significantly higher in African population compared to American population with an African ancestry (Kaplan *et al.*, 2008). Previous studies reported that neonatal jaundice is associated with both environmental and genetic factors (Kaplan and Hammerman, 2005; Behjati-Ardakani and Sedaghat, 2007).

In Malaysia, cord blood sampling of infants are screened for haemolysis sign and ABO incompatibility (Fabris *et al.*, 2009). According to Bosma 1995, variants in *UGT1A1* gene play a critical role towards the development of neonatal jaundice such

as the A(TA)7TAA in the promoter region. In 1995, a study reported that variants in the Uridine Diphosphate Glucuronosyltransferase 1A1 (*UGT1A1*) gene is a potential as a genetic risk factor for neonatal hyperbilirubinemia (Bosma *et al.*, 1995). For instance, serum bilirubin levels in Japanese newborns were higher as compared to Caucasian newborn infants due to different genetic background between populations (Fischer *et al.*, 1988).

1.2 Uridine Diphosphoglucuronate Glucuronosyltransferase (UDPGT) 1A1 gene

The entire structure of uridine diphosphoglucuronate glucuronosyltransferase 1A1 (*UGT1A1*) gene was first reported in 2001 (Gong *et al.*, 2001). Van Es et al found that the *UGT1A1* gene complex is located on chromosome 2q37 (Figure 1.1)(Van Es *et al.*, 1993). The length of this gene is 218 kb. It consists of 13 amino terminal exons and four common carboxy terminal exons. The amino terminal exons are responsible to encode the substrate binding domain where as the carboxy terminal exons are responsible to encode the UDP glucuronic acid binding and membrane spanning region. The presence of variants in this gene results in a frameshift or a premature stop codon and produce non-functional protein (Bosma, 2003).

This gene is responsible for the production of the enzyme bilirubin uridine diphosphate glucuronosyltransferase (B-UGT) that is important in glucuronidation activity (Ritter *et al.*, 1991). Polymorphisms in the exons and promoter region of the *UGT1A1* gene will cause structural or functional abnormalities of the enzyme which leads to non-hemolytic unconjugated hyperbilirubinemia (Kaplan *et al.*, 2003). Crigler Najjar syndrome type 1 (CN-I, a severe form), Crigler Najjar syndrome type 2 (CN-II, an intermediate form) and Gilbert syndrome (GS, a mild form) is

associated with variants in the *UGT1A1*gene, based on clinical severity (Seppen *et al.*, 1994; Kadakol *et al.*, 2000).

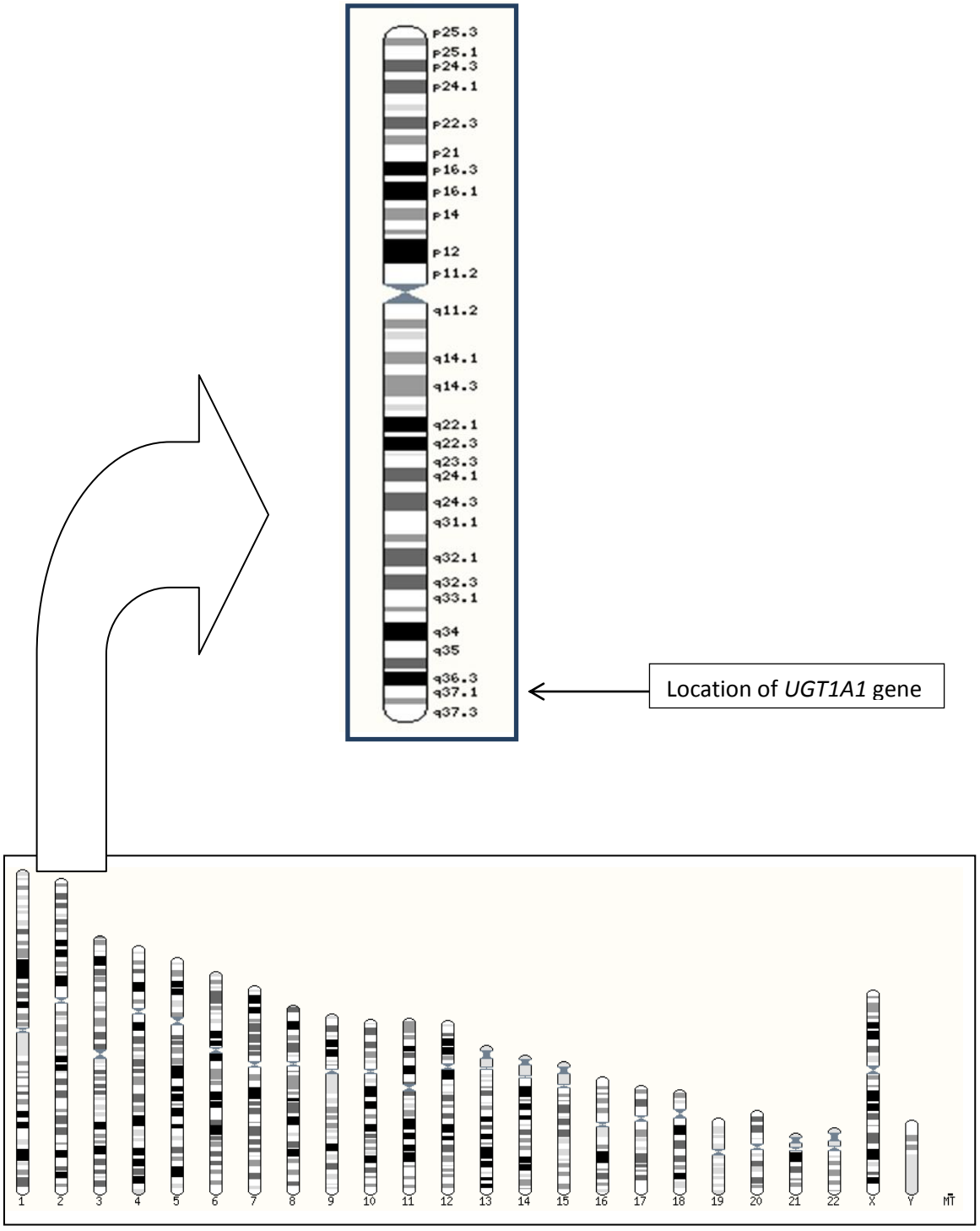

Figure 1.1: Location of the *UGT1A1* gene in chromosome 2(2q37.1)

:http://asia.ensembl.org/Homo_sapiens/Location/Chromosome?R=2:234669368-234682419;g=ENSG00000241625;r=2:226432585-226432874;t=ENST00000466851

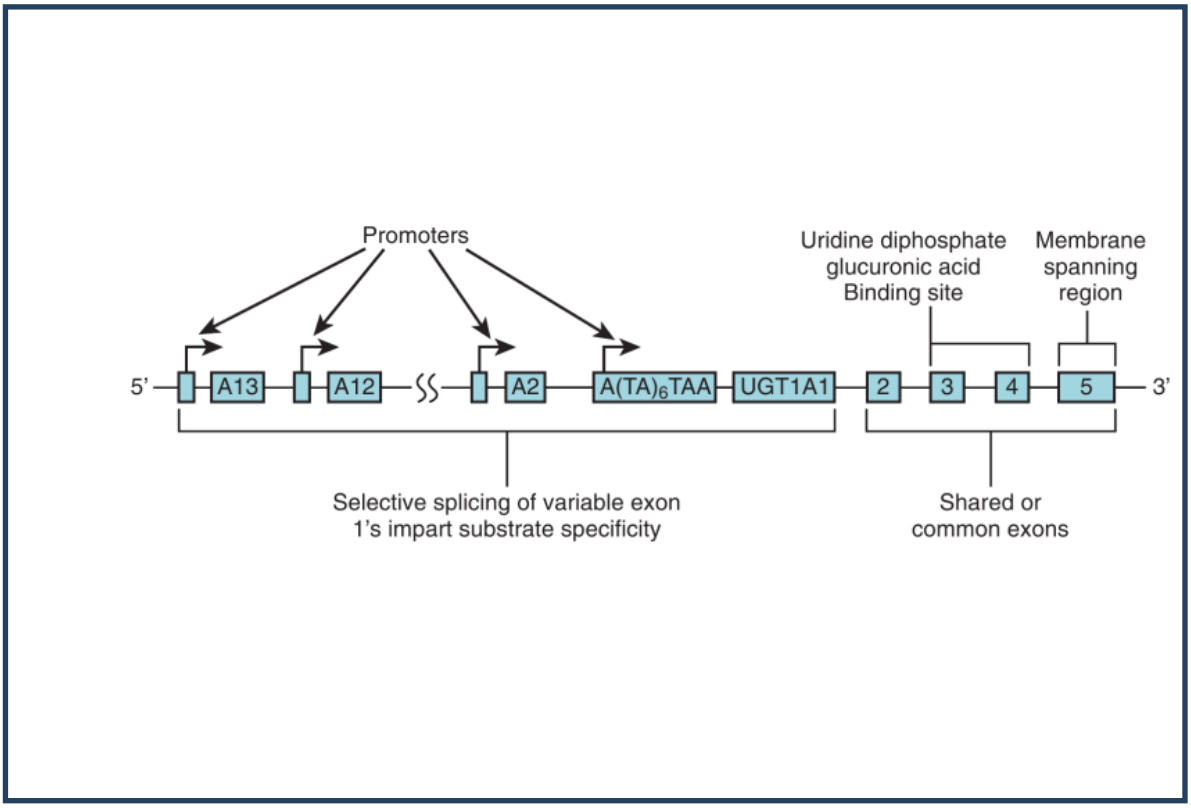

Figure 1.2: Sites for human uridine diphosphate glucuronosyltransferase-1 gene (UGT1). UDPGA, uridine diphosphate glucuronic acid binding site and membrane spaning region.

Gourley GR. Neonatal jaundice and disorder of bilirubin metabolism. Suchy FJ, Sokol RJ, BalisteriWF [Eds]. Liver disease in children, 3rd ed. New York, Cambridge University Press, 2007

Liver is the major organ that is responsible for glucuronidation during the oral absorption of drug in the body (Tukey and Strassburg, 2000; Fisher *et al.*, 2001; Strassburg, 2008). In human, bilirubin molecule is one ofthe endogenous substrates that is selectively glucuronidated by the superfamily of enzymes human UGTs of *UGT1A1* gene. Whereas drugs, environmental pollutants, chemical carcinogen and dietary substances are examples of exogenous substrates for human UGT (Tukey and Strassburg, 2000)(Ritter, 2000).

The exogenous substrates such as drug molecules are hydrophilic and easily excreted (Ouzzine et al., 2003). In the glucuronidation reaction, UDP glucuronic acid is converted to hydrophobic drug molecules to form β-D-glucuronides which comprise of functional group; that consist of aromatic, aliphatic alcohols, carboxylic acid, amines and free sulfhydryl moiety (King et al., 2000, Guilemette, 2003). Variant of A(TA)7TAA located in the promoter region of the *UGT1A1* gene is one of the commonest genetic polymorphism associated with Gilbert syndrome in Caucasian population. Previous studies suggested that the *UGT1A1* gene is responsible for glucuronidation activity in the bilirubin metabolism pathway (Miners et al., 2002; Burchell, 2003).

The A(TA)7TAA variant is associated with the reduction and elimination of active metabolite of irrinotecan, SN-38 (Ando et al., 2000, 2002; Iyer et al., 2002). A study in 2004 suggested that, variants of A(TA)7TAA may result in drug toxicity. The detection of this variant, facilitates the use of alternative therapy in reducing the incidence of irrinotecan toxicity (Marsh & McLeod, 2004). In other studies, it was suggested that, Crigler Najjar syndrome patients are associated with homozygous variant in the TATA box in the promoter region of the *UGT1A1* gene (Kearns et al., 1985; Bosma 2003).

Based on Innocenti et al in 2005, they found that variant TATA box, c.211G>A and c.-3279T>G are the three major variants with different prevalence in various population. In year 1993, Van Es et al showed that variant TATA box was found in patient with Crigler Najjar type II and Gilbert syndrome. It is located in the proximal promoter region which the variant consist of an additional TA insertion, A(TA)7TAA. A few years later, Ciotti et al showed that the variant TATA box is also found in Crigler Najjar syndrome type I (Ciotti et al., 1998).

The development of Gilbert's syndrome is not entirely dependent on the A(TA)7TAA variant. For example, homozygosity of A(TA)7TAA in most Gilbert syndrome patients may occur in combination homozygosity of the other variant, c.-3279T>G (Maruo et al., 2004; Costa et al., 2005; Ferraris et al., 2006).

The 2-bp addition in the TATA box promoter region was reported to reduce the *UGT1A1* gene expression level of *UGT1A1* gene in Gilbert syndrome patients by 25 to 50% of normal (Bosma et al., 1995; Monaghan et al., 1996a). A few years later, Bancroff and Monaghan discovered that homozygosity of A(TA)7TAA can accelerate neonatal jaundice (Monaghan et al., 1996b; Bancroft et al., 1998).

Other than A(TA)7TAA, there are two types of polymorphic variants in the *UGT1A1* promoter region which are c.-3279T>G and c.-3156G>A. The c.-3279T>G variant is located in phenobarbital responsive enhancer module (gtPBREM) (Sugatani et al., 2001) and was postulated to reduce the responsiveness of *UGT1A1* transcription to bilirubin (Yusoff et al., 2010). It reduced 60% activity of *UGT1A1* promoter and was suggested as a genetic risk factor for neonatal jaundice (Sugatani et al., 2002; Yusoff et al., 2010)

1.3 Gilbert syndrome

Gilbert syndrome is caused by variants in the *UGT1A1* gene. In Gilbert syndrome patients, the hepatic activity of bilirubin glucuronosyltransferase is decreased to 30% of normal which can reduce the percentage of bilirubin diglucuronides in bile (Arias and London, 1957; Black and Billing, 1969; Fevery *et al.*, 1977). The genetic basis of the reduction of bilirubin glucuronidation was discovered by Bosma in 1995 (Bosma *et al.*, 1995) and varied between different population (Beutler *et al.*, 1998; Hall *et al.*, 1999).

According to previous studies, the percentage of Gilbert syndrome in Caucasian population was 5 to 10% and is a common disorder (Owens and Evans, 1975; Sieg A, 1987). In this population, the bilirubin glucuronidation activity was reduced by 80% in patients who were homozygous variant for TA insertion in TATA box in the proximal promoter of the *UGT1A1* gene (Bosma, 2003). The efficiency of transcription of the *UGT1A1* gene is reduced when the expanded element seven TA repeats were present. According to previous data, increasing number of TA repeats leads to decreasing transcriptional activity in the *UGT1A1* gene promoter (Iolascon et al., 1999). This report was supported by Bosma and Monaghan that homozygosity of A(TA)**7**TAA in Gilbert syndrome can reduce the expression level activity of the *UGT1A1* gene.

Gilbert syndrome affects 6 to 12% of individuals which their range of serum bilirubin levels is between 52 and 86 μmol/L. When the serum bilirubin level in patients with Gilbert syndrome is increased, it can cause reduction in hepatic activity of bilirubin glucuronsyltransferase (Arias and London, 1957; Black and Billing,

1969). Previous studies suggested that most of heterozygous missense variants of *UGT1A1* gene were related to A(TA)7TAA variant.

1.4 Crigler Najjar syndrome

In 1992 and 1993, the first variant causing Crigler Najjar syndrome type I and type II were discovered (Bosma *et al.*, 1992; Ritter *et al.*, 1992; Bosma PJ, 1993). The frequency of this syndrome is very rare which is 0.6 per million (van der Veere et al., 1996). Crigler Najjar syndrome is a type of *UGT1A1* enzyme deficiency syndrome. It can be divided into two types which is type I and type II. Crigler Najjar type I (severe phenotype) and type II (intermediate phenotype) is the result from complete and partial deficiencies of *UGT1A1* gene activity (Owens and Evans, 1975). Some of Crigler Najjar syndrome relatives have mild form unconjugated hyperbilirubinemia has been reported in 1962 (Arias, 1962).

Phenobarbital treatment was suggested to cause 30% reduction of serum bilirubin level in CN type II but no minor effect is observed in CN type I. The traces of bilirubin glucuronides for CN type I patient was absent and it was present in CN type II patient. The presence of bilirubin glucuronides in bile indicate bilirubin glucuronidating activity. Some of CN type II patients have died due to kernicterus (chronic bilirubin intoxication in the brain) but most will survive until adulthood.

Diagnosis of neonatal jaundice

	Gilbert syndrome	Crigler Najjar Syndrome type I	Crigler Najjar syndrome type II
Prevalence	3%	rare	rare
Inheritance	Autosomal dominant or recessive	Autosomal resessive	Autosomal recessive,rarely dominant
Genetic Defect	*UGT1A1* gene	*UGT1A1* gene	*UGT1A1* gene
Hepatocyte defect site	Microsomes±plasma membrane	Microsomes	Microsomes
Deficient hepatocyte function	Glucuronidation±uptake	Glucuronidation	Glucuronidation
BUGT activity	5-53% of controls	Severely decrease	2-23% of controls
Total serum bilirubin levels (mg/dL)	0.8-4.3	15-45	8-25
Serum bilirubin decrease with Phenobarbital(%)	70	0	77
Bile bilirubin conjugates			
Diglucuronide(normal~80)	60	0 to trace	5-10
Monoglucuronide(normal~15)	30	Predominant if measurable	90-95
Prognosis	Benign	Kernicterus common	Occasional kernicterus

Table 1.1: Comparison disorder of unconjugated hyperbilirubinemia.Gourley GR. Neonatal jaundice and disorder of bilirubin metabolism. Suchy FJ, Sokol RJ, BalisteriWF[Eds]. Liver disease in children, 3rd ed. New York, Cambridge University Press, 2007

1.5 Bilirubin Uridine Diphosphate Glucuronosyltransferase (B-UDPGT)

Bilirubin uridine diphosphate glucuronosyltransferase (B-UDPGT) enzyme is the catalyst that is responsible for bilirubin glucuronidation pathway. Its deficiency can lead to unconjugated hyperbilirubinemia (Bosma et al., 1994). The percentage of activity towards bilirubin changes with age. These enzyme activity is shown as 0.1 in middle fetal, 0.1 to 1 during neonatal and 1 to 100% in early infatile respectively, by refering with mature phase values, (Kawade and Onishi, 1981). The bilirubin levels also correlate with the frequency of different variants either in the promoter or coding regions of the *UGT1A1* gene (Huang *et al.*, 2000). B-UDPGT is encoded by 5 exons including 1A1, 2, 3, 4 and 5 at the UGT1A locus (Van Es *et al.*, 1993). According to a report in the year 2003, the nomenclature of UGT1A is based on 5 respective exons (Mackenzie et al., 2003). The 4 common exons are involved in the co-substrate and substrate binding domain due to C-terminal and N-terminal portion of the enzyme (King *et al.*, 2000). The *UGT1A* gene locus contains 12 variable exons (A2-A13) that are responsible to encode other UDPGT isoform (Clarke *et al.*, 1997).

Bilirubin is the product from heme catabolism. Heme is found from hemeproteins, haemoglobin, cytochromes, catalase and peroxidase. Bilirubin is changed to bilirubin glucuronic acid in the liver and will be excreted in bile (Bosma, 2003). There are two kinds of bilirubin in man which is direct reacting bilirubin and indirect reacting bilirubin.

1.51 Indirect Hyperbilirubinemia (Unconjugated Bilirubin)

Unconjugated bilirubin causes indirect hyperbilirubinemia. It occurs when the serum bilirubin level is greater than 34.2µmol/L and it consists of lipid soluble molecule. With these, bilirubin is able to accumulate in certain parts of the body. Only a small fraction of unconjugated bilirubin that is unbound to albumin can enter the brain. Accumulation of bilirubin in the brain may cause neurological disease and kernicterus. It occurs due to the ability of bilirubin to cross the blood brain barrier (Juretschke, 2005). The presence of endogenous or exogenous binding competitors compound such as drug towards albumin decreases the binding affinity of albumin resulting in kernicterus.

1.52 Direct Hyperbilirubinemia (Conjugated Bilirubin)

Direct hyperbilirubinemia is caused by conjugated bilirubin. Conjugated bilirubin is water soluble molecules that are able to move from the bile duct to the intestine (Odell, 1980). This conjugation process is catalysed by uridine glucuronosyltransferase (UGT). According to Klein, hepatocellular disease and metabolic disorder are general factors for direct hyperbilirubinemia in the neonates (Klein et al., 2010).

Due to erythrocyte cycles, bilirubin is produced at 250mg/day. This mechanism is potentially associated with jaundice and hyperbilirubinemia (Chodhury R et al., 1995). In human, bilirubin diglucuronide is catalyzed by bilirubin UDP-glucuronosyltransferase in hepatic endoplasmic reticulum and efficiently transported into the liver because of more polar water soluble of bilirubin diglucuronide. It is then excreted into the bile via membrane in the bile canaliculus (Clark D J et al., 1997).

1.53 Redox cycle

Heme oxygenase and biliverdin reductase consist of reducing properties that are responsible for degradation of heme and reduction of bilirubin. Bilirubin has antioxidant potential especially in blood plasma, as demonstrated by many chemical and biochemical in vitro studies and it can protect the cell from oxidative damage. However, this hypothesis remains unclear. Redox cycle is the famous explanation for antioxidant properties for bilirubin. These cycles showed that biliverdin were recycled by bilirubin reductase following oxidation by bilirubin (Maghzal G J et al., 2009). This enzyme has an important role against H_2O_2-mediated oxidative stress through redox amplification cycle (Sedlak T W et al., 2004). Redox cycle occurs in the presence of the ubiquitous of biliverdin reductase (Maghzal et al., 2009). In the presence of intramolecular hydrogen bonding inside the bilirubin molecules, it became soluble in water at physiological pH and ionic strength (McDonagh et al., 1979).

These hydrogen bond exist when bilirubin is combined with cell membrane (Nogales D et al., 1995). Before the uptake of bilirubin by hepatocytes, it is closely bound to albumin and will be circulated within the blood (Brodersen et al., 1980). Inside the hepatocytes, bilirubin molecules are attached with glutathione S-transferases before being converted to water soluble derivatives. This process involves conjugation of one or both of its propionyl groups before its discharge into bile and the intestine (Fevery Jet al., 1977). When the molecule of bilirubin is attached to the primary site of albumin, it were leading to formation of two planar dipyrroles involve ion pairing, hydrogen bonding and π interaction out of plane position and leading to break up its intramolecular hydrogen bond (Hsieh Y Z 1988).

Conjugated bilirubin is hydrophilic which is easier to be excreted into bile. In the absence of these enzyme, bilirubin cannot be excreted rapidly by the liver. In these cases, serum bilirubin will increase and bilirubin molecules is deposited in the body tissue leading to neonatal hyperbilirubinemia. Unconjugated bilirubin in the serum may cross the blood brain barrier (BBB) and take them at risk of developing bilirubin encephalopathy (acute bilirubin intoxication in the brain) or kernicterus (chronic bilirubin intoxication in the brain) Bhutani and Johnson, 2005; Morioka., 2010). The complete scheme of the biosynthesis of bilirubin is shown in Figure 1.3.

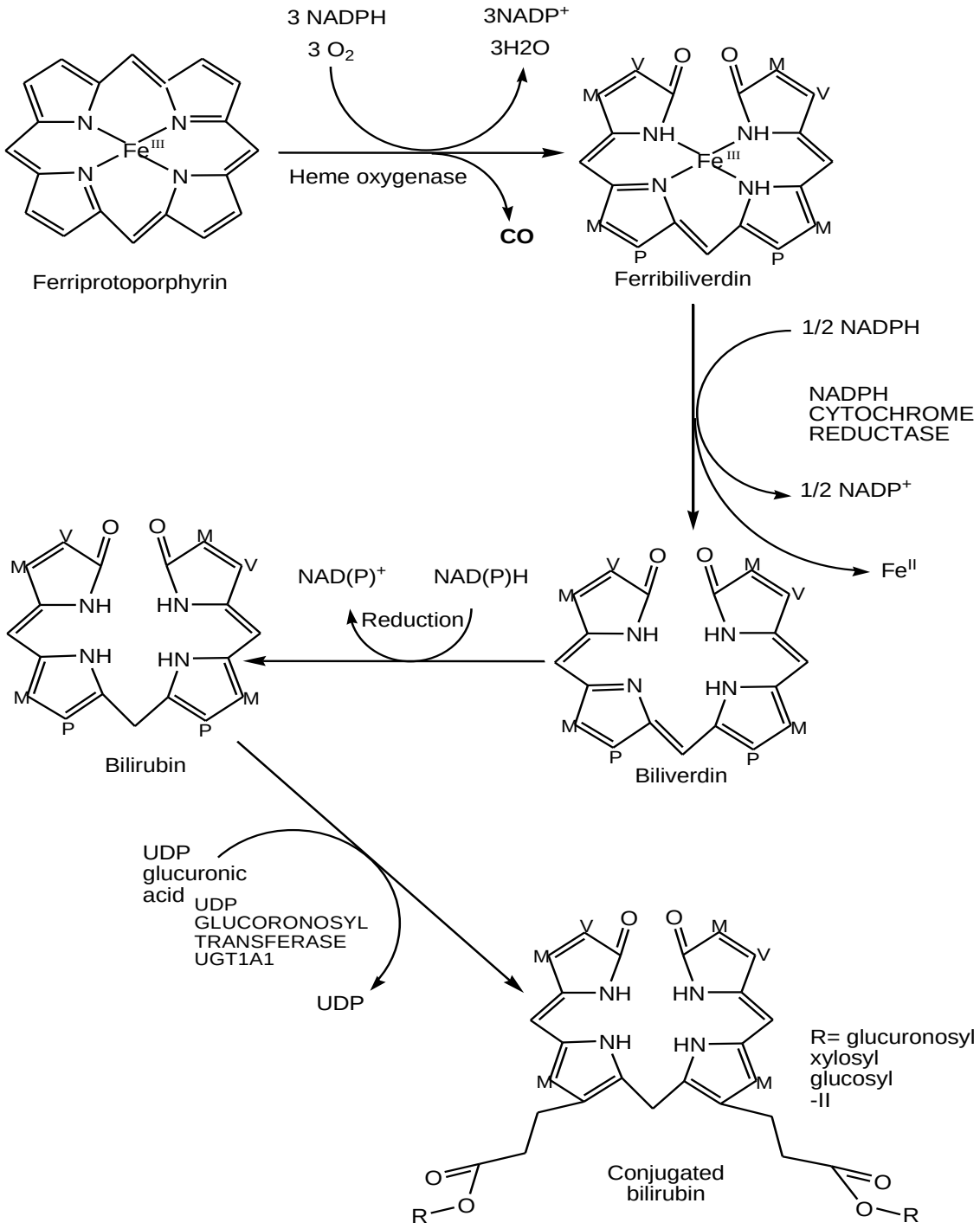

Fig. 1.3: The complete scheme of the stereochemistry biosynthesis of bilirubin metabolism. The production of bilirubin from heme catalyse by heme-oxygenase and subsequently biliverdinreductase. Glucuronidation of bilirubin by UDP-glucuronosyltransferase*UGT1A1* results in the formation of bilirubin mono- and di-glucuronide which are excreted into bile. The figure was adapted from Bosma, 2003

1.6 Mechanism of Phototherapy

Phototherapy is an effective treatment in infants to decrease the concentration of circulating bilirubin level. It has been used as a method of treating neonatal hyperbilirubinemia because of the effectiveness of light and its ability to reduce serum bilirubin during phototherapy (Maisels and McDonagh., 2008). A previous study suggested that intensive phototherapy is able to reduce 10mg per decilitre of serum bilirubin levels within a few hours in infants who have serum bilirubin levels greater than 30mg per decilitre (Hansen, 1997). Phototherapy refers to the use of light to convert bilirubin molecules in the body into water soluble isomers that can be excreted by the body. This treatment is effective due to the ability of blue light to break the internal Hydrogen bonds in unconjugated bilirubin molecules namely photoisomer. The product forms are hydrophilic molecules that are soluble in water which can be excreted in bile (Van der Veere *et al.*, 1997).

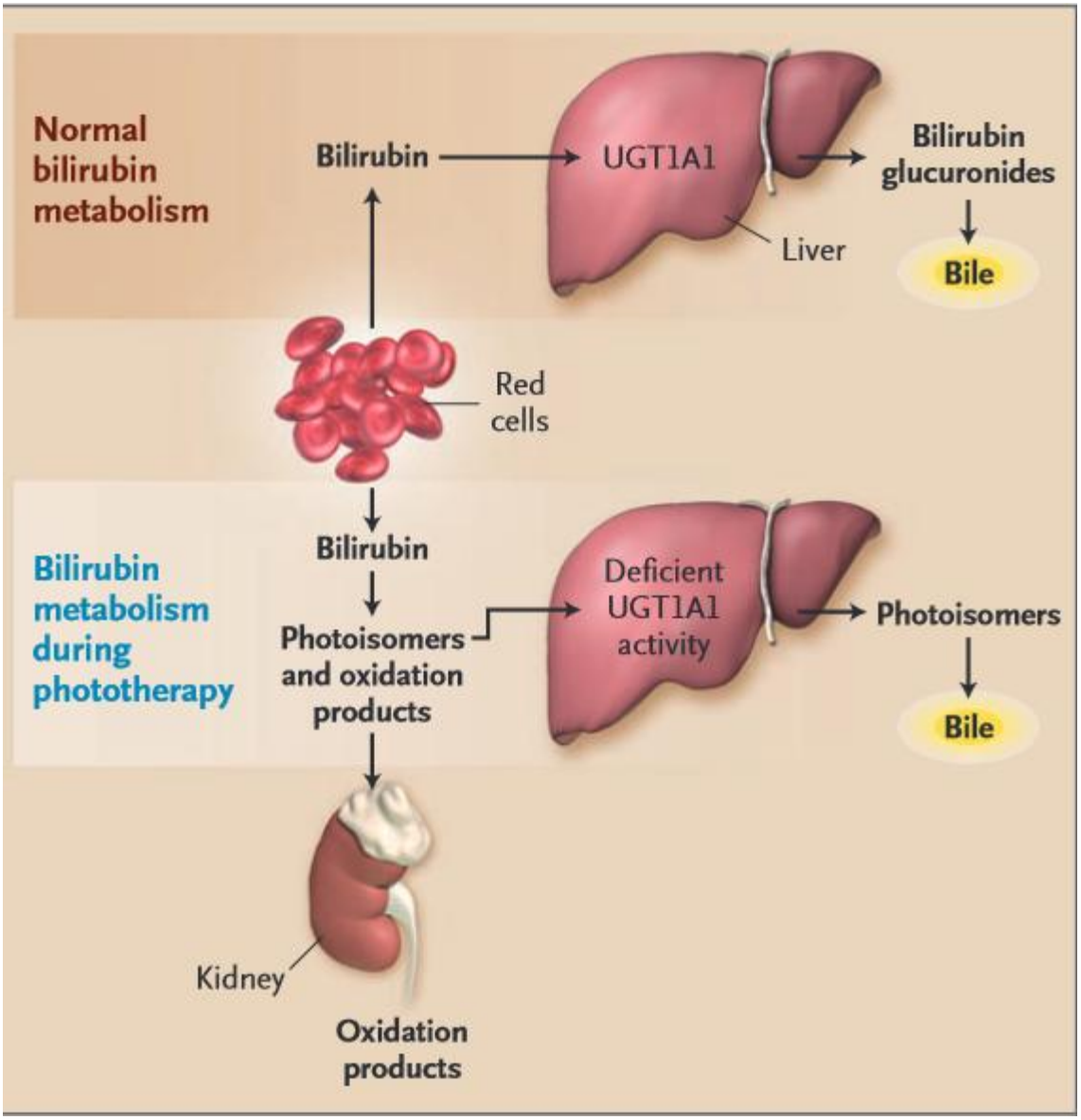

Figure 1.4: Biological pathway of normal bilirubin metabolism with and without phototherapy (Maisels and McDonagh, 2008)

This mechanism is effective because three reactions can occur when bilirubin is exposed to light. Absorption of light by the dermis will change the normal structure of bilirubin form 4*Z*, 15*Z*-bilirubin into 2 isomeric forms: structural and configurational isomer. Both *Z* and *E* configurationsand prefixes 4 and 15 are used to indicate the stereochemistry and position of double bond respectively (McDonagh, 1986).

The main structural isomer of bilirubin is *Z*-lumirubin and the main configurational isomer of bilirubin is 4*Z*, 15*E* –bilirubin. Configurational isomer is reversible but structural isomer is irreversible. Both photo isomers are less lipophilic than normal bilirubin and can be excreted into bile without undergoing glucuronidation in the liver. Some of the configurational isomers of bilirubin revert spontaneously to the parent form isomer after excretion into bile and can be reabsorbed via the enterohepatic circulation in the gut. Structural bilirubin isomers, consist of intramolecular cyclization resulting in *Z*-lumirubin, which can also be excreted in the urine.

The absorption of light by normal form bilirubin also results in the generation of excited-state bilirubin molecules that react with oxygen to produce colourless oxidation products. This process occurs more slowly than configurational or structural isomerization. The figure 1.5 below provides a schematic of the conversion of normal bilirubin to configurational isomers, structural isomers and photooxidation products and the respective routes of excretion from the body (McDonagh, 1986).

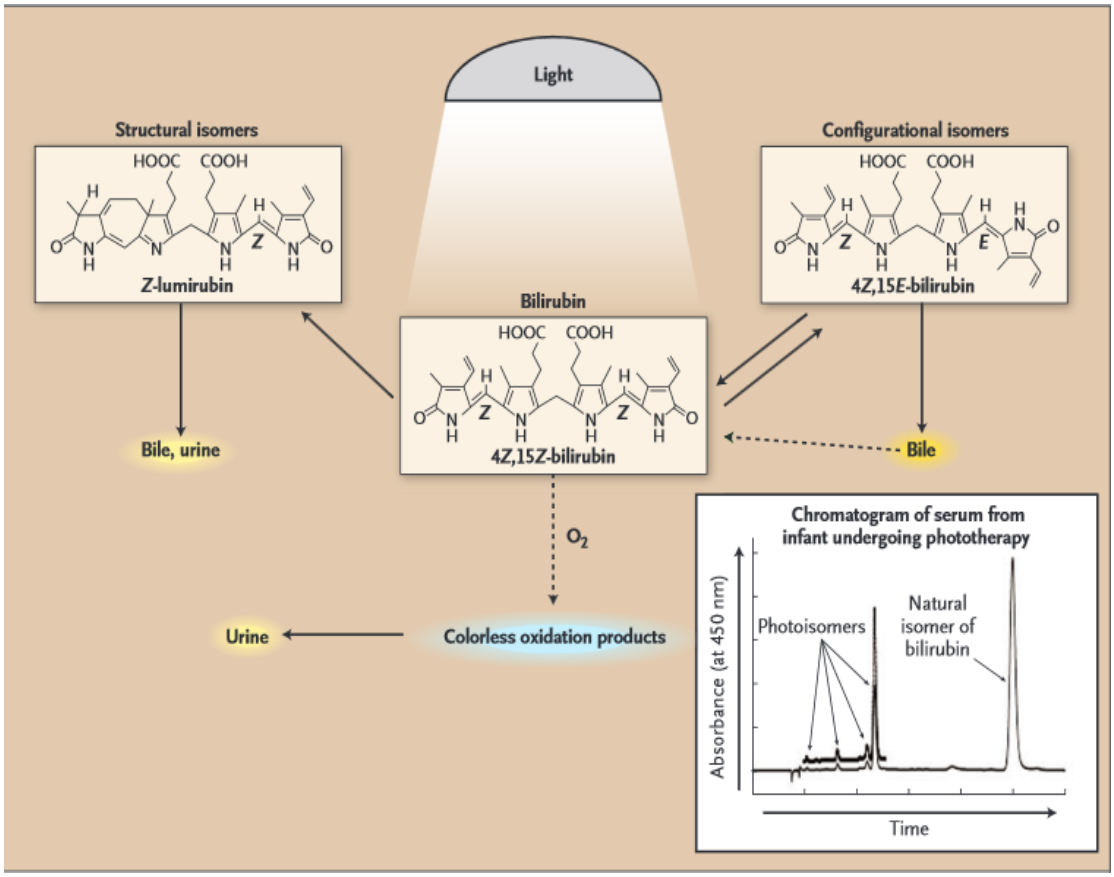

Figure 1.5: Mechanism absorption of light by normal form of bilirubin (McDonagh, 1986)

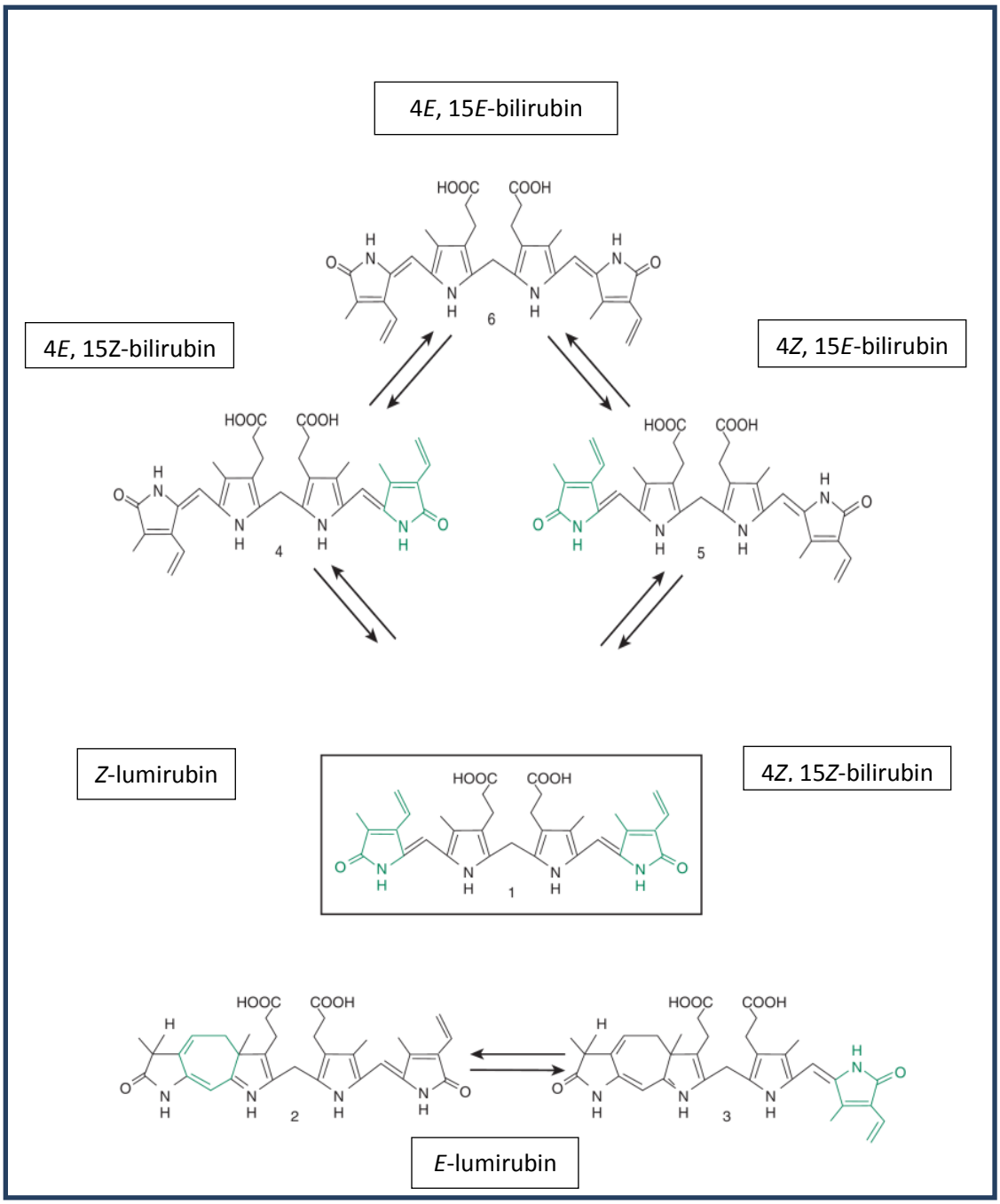

Figure 1.6: The stereochemistry of 2 isomeric form; structural and configurational isomer structure when absorbtion of light by dermal surface.Taken from: Gourley GR. Neonatal jaundice and disorder of bilirubin metabolism.Suchy FJ, Sokol RJ, BalisteriWF[Eds]. Liver disease in children, 3rd ed. New York, Cambridge University Press, 2007

In phototherapy, blue-green light mechanism in the range of 460-490 nm is the most effective for treating hyperbilirubinaemia. The absorption of light by one or both of the propionic acid side chains in the normal bilirubin (4*Z*, 15*Z*-bilirubin) can increase the polarities which generates configuration isomers, structural isomers, and photooxidation products. The two principal photoisomers formed in human are shown in Figure 1.5. Photooxidation occurs more slowly than configurational and structural isomerization. Photooxidation and photoisomer products are primarily excreted in the urine and bile (Maisels and McDonagh, 2008).

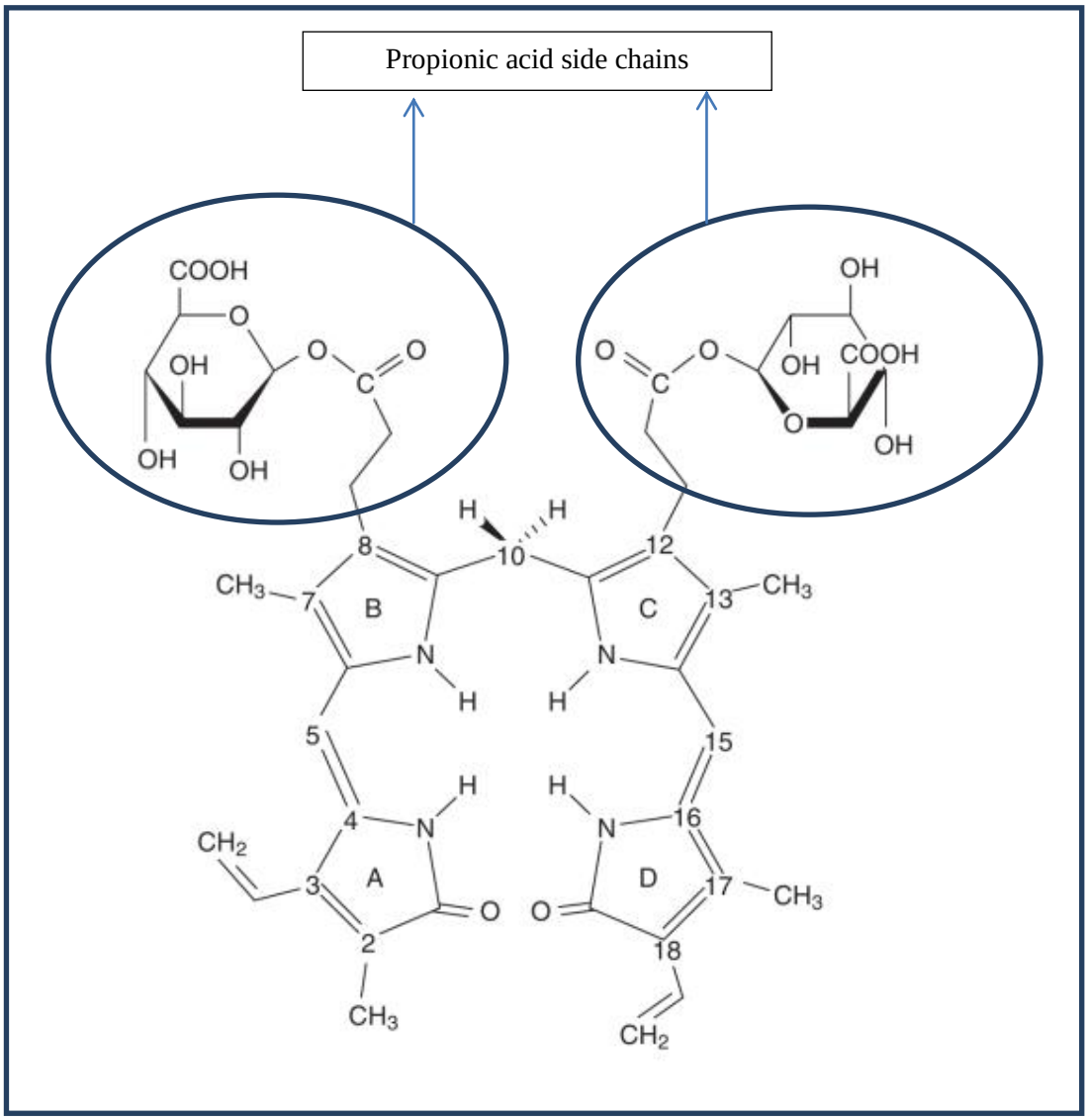

Figure 1.7: Bilirubin diglucuronide. In bilirubin monoglucuronide, only one propionic acid side chain (C-8 or c-12) is glucuronidated. Taken from: Gourley GR. Neonatal jaundice and disorder of bilirubin metabolism. Suchy FJ, Sokol RJ, BalisteriWF[Eds]. Liver disease in children, 3rd ed. New York, Cambridge University Press, 2007

1.7 Bilirubin metabolism

Bilirubin is the oxidative product that is produced by catabolism of hemoglobin. Hemoglobin is the largest source of bilirubin production. The hemoglobin catabolism and bilirubin production are higher in newborn neonates compared to older children and adults due to shorter life span of red blood cells of 70 to 90 days. The bilirubin clearance is delayed if there is immaturity of hepatic glucuronosyltransferase and inadequate milk intake (Moerschel *et al.*, 2008). More than 80% of the bilirubin produced is derived from heme catabolism of senescent red blood cells (RBC) and the remaining are derived from other hemoproteins such as myoglobin and cytochrome P-450 (Kaplan and Hammerman, 2005). In the initial stage, bilirubin is in the unconjugated form and is lipid soluble. This molecule enters the serum and is transported to the liver and binds to albumin (Moerschel et al., 2008). According to Kaplan 2005, sufficient serum albumin should be available with the amount of bilirubin produced under physiological conditions and unbound bilirubin freely accumulates in the serum. Conjugated molecule of bilirubin albumin in the newborn babies against bilirubin encephalopathy.

The production of bilirubin, it is started by heme oxygenase which is involved in the heme catabolism in mammalian cells. In the reticuloendothelial system, heme is broken down into biliverdin and latter subsequently metabolized to bilirubin. These process are catalyzed by enzymes hemeoxygenase and bilirubin reductase. For each molecule of bilirubin derived by this process, equimolar quantities of carbon monoxide (CO) are being produced. These CO molecule binds to hemoglobin to form carboxyhemoglobin (COHb) which it is transported to the lungs and exhaled (Bosma, 2003). Biliverdin produced will be converted to bilirubin by catalytic enzyme, biliverdin reductase in the presence of NADPH. Elevation

yields of biliverdin are limited in certain oxidants such as peroxyl radical and albumin bound bilirubin (Maghzal et al., 2009).

Unconjugated bilirubin is an organic anion and toxic to the central nervous system and it cannot be excreted in this form (Kaplan and Hammerman, 2005; Moerschel *et al.,* 2008). To begin the excretion process, unconjugated bilirubin is taken up by hepatocyte. Unconjugated bilirubin is conjugated into glucuronic acid which is water soluble and easily excreted by the liver and biliary tract. This process is catalyzed by enzyme uridine diphosphoglucuronate glucuronosyltransferase 1A1 (*UGT1A1*). Monoglucuronide and diglucuronide fractions are the identified form of conjugated bilirubin fraction. For monoglucuronide fraction, bilirubin reacts with one glucuronic acid whereby for diglucuronide fraction bilirubin reacts with two glucuronic acids. Both fraction product are excretable and most are converted to non-reabsorble products and excreted in the stool (Kaplan and Hammerman, 2005).

Based on Sarah and co-workers, bilirubin will be reabsorbed by the intestine if it is converted back into the unconjugated form. In the breastfed neonates, the mucosal enzyme β-glucuronidase can cleave off the conjugated bilirubin molecule resulting in unconjugated bilirubin molecule to be reabsorbed, re-entering the bilirubin pool and loaded on the hepatocyte. This is known as the enterohepatic bilirubin circulation.

Biliverdin is a linear tetrapyrole form and the product of protoporphyrin (tetrapyrrole ring of protoheme) in the rate limiting step that is catalysed by microsomal hemeoxygenase enzyme (Ryter *et al.,* 2006). HO consists of two major form, inducible form (HO1) and constitutive form (HO2). HO1 is located in the spleen, liver and bone marrow and HO2 is located in the testes, central nervous